Docket No.: 0933-0210P
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Mart SAARMA et al.

Application No.: 10/648,361

Confirmation No.: 3435

Filed: August 27, 2003

Art Unit: 1633

For: NOVEL NEUROTROPHIC FACTOR PROTEIN AND USES THEREOF

Examiner: M. Marvich

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Mart Saarma, hereby declare as follows:

I am a citizen of Estonia, residing at Helsinki, Finland (Home address: Kulosaaren puistotie 38A4, 00570, Helsinki, Finland).

I am presently employed as the director and professor at the Institute of Biotechnology, University of Helsinki.

A copy of my Curriculum Vitae is attached.

I am a co-inventor of the subject matter of the above-identified U.S. Patent application. I am familiar with the specification and pending claims, and with the prosecution history of the application.

The Examiner has rejected claim 7 of the application as allegedly lacking utility and not providing an enabling disclosure. The Examiner asserts that the claimed invention is not supported by a specific and substantial utility. The Examiner agrees that the specification discloses the use of the claimed polypeptide as a treatment for Parkinson's disease, but states that further research is necessary to reasonably confirm a "real world" use.

First, the allegation of utility of the invention for treatment of Parkinson's disease or other neurodegenerative disease is consistent with the data of Figure 8, showing expression of MANF2 mRNA in the thalamus, cortex and the hippocampus. Figure 5 data show MANF2mRNA expression in the following regions of the human brain: hippocampus, thalamus, amygdala, corpus callosum, cerebellum, caudate nucleus, cerebral cortex and substantia nigra Such an allegation is also supported by the data in Figures 10-12 that show that MANF2 supports survival of dorsal root ganglion and dopaminergic cells in culture.

Second, I have determined that the specification describes a working example for determining the dosage of MANF2 useful to treat Parkinson's disease and other neurological disorders. The specification describes a number of techniques for administering MANF2 to the brain and includes pre-termed dosage levels, *e.g.* at p 53, line 18 to page 54, line 19. Also, the specification discloses several animal models by which an artisan of ordinary skill may evaluate any particular mode of therapy chosen can be evaluated for efficacy. (Example 8, page 67 of the specification as filed).

In view of the above, any experimentation alleged by the Examiner must be deemed well-guided, not undue, and therefore, the alleged utility of treatment of neurodegenerative disease, as exemplified by Parkinson's disease should be considered enabled by the specification.

To demonstrate that such is the case, I describe here, using the guidance from Example 8 in the specification as filed, the effects of a 10 µg dosage of MANF2 on the rat 6-hydroxydopamine (6-OHDA) model of Parkinson's disease. The results show that MANF2 can rescue midbrain dopaminergic neurons *in vivo*. Therefore, MANF2 may be used to treat diseases characterized by the degeneration of dopaminergic neurons, such as Parkinson's disease, as asserted in the specification as filed, *e.g.* at page 43, line 16.

<u>Experimental Design</u>

All rats were exposed to a stereotaxic microinfusion twice in the left dorsal striatum; first they were given either 4 µl of phosphate-buffered saline (PBS), 10 µg of glial cell line-derived neurotrophic factor (GDNF), or 10 µg of MANF2, the dosage described in the specification. The coordinates in the left striatum relative to the bregma and dura were A/P + 1.0, L/M + 2.7, D/V – 4, according to the atlas of Paxinos and Watson, 1986 (Paxinos, G. & Watson, C., 1986. *The Rat Brain in Stereotaxic Coordinates*, Academic press, San Diego.) Six hours later, each animal received 8 µg of 6-OHDA in the same site in the left dorsal striatum as described above for PBS, GDNF or MANF2.

Rotational behavior

Behavioral test were carried out 2 and 4 weeks post-lesion. The rats were allowed to

habituate to the test chamber for 30 min before D-amphetamine (University Pharmacy, Helsinki, Finland; 2.5 mg/kg i.p) was administered. The number of full (360°) ipsilateral and contralateral turns was recorded for a period of 2 h. Net ipsilateral turns to the lesion were calculated by subtracting the turns to the left from the turns to the right.

Immunohistochemistry

At 4 weeks post-lesion, the rats were anesthetized with an overdose of natriumpentobarbital (90 mg/kg, i.p. Orion Pharma, Finland) and perfused intracardially with PBS followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The brains were removed, post-fixed for 4 h, and stored in sodium phosphate buffer containing 20% sucrose at 4°C. Serial coronal frozen sections of 4μm were cut on a sliding microtome. Six sets of sections were collected in cryoprotectant solution (0.5M PB, 30% glycerol and 30 % ethylene glycol) and stored at –20°C until immunohistochemical processing. Free-floating sections were processed for tyrosine hydrolase (TH)-immunohistochemistry. Following three rinses in PBS, endogenous peroxidase activity was quenched for 5 minutes in 3% H_2O_2/10% methanol/PBS. After 3 rinses in PBS, sections were pre-incubated with normal horse serum (NHS/0.3% Triton X-100 in PBS) in order to block nonspecific staining. Thereafter, sections were incubated overnight at room temperature with 1:2000 dilution of biotinylated mouse-anti-TH (Chemicon, Temecula, CA). This incubation was followed by incubations with 1:200 dilution of biotinylated horse-anti-mouse (Vector, BA2001) and by incubation in the avidin-biotin peroxidase complex using the Elite ABC vectastatin kit (Vector Laboratories). The reactions were visualized using DAB as a chromogen.

Morphological Analysis

SN cell counts

Unbiased stereological cell counting procedures were used to count TH-positive cells in the substantia nigra *pars compacta* (SNpc) by using the optical fractionator method in combination with the dissector principle and unbiased counting rules (West et al., 1991, *Anat. Rec.* **231**, 482-497; Mouton et al., 2002, *Brain Res.* **956**, 30-35). The entire SNpc was analyzed with a Stereo Investigator platform (MicroBrightField, Germany) attached to an Olympus BX51 microscope. From each animal, 3 sections from the central portion of the SNpc, where the medial terminal nucleus (MTN) was present (level A/P –5.3), were selected for quantitative analysis. Optical fractionator estimation method was optimized to give a coefficient of error less than 15% per individual brain sample. Each reference space was outlined at low power (4X), and cells were counted using a high magnification (60X, oil immersion) objective.

<u>Results</u>

Behavioral tests

Behavioral tests were carried out twice in all rats. Two and four weeks post lesion, each rat was given D-amphetamine (2.5 mg/kg, i.p.) in order to induce ipsilateral (to the side of lesion) turning behavior, which was recorded for a period of 2 h. At two weeks post lesion, amphetamine did induce significant ipsilateral turning behavior in the control group. However, no increase in ipsilateral turns was observed in the treatment group. At four weeks post-lesion, MANF2 was not able to significantly reverse the amphetamine-induced ipsilateral turning.

TH-immunohistochemistry

At four weeks post lesion, following the second behavioral experiment, the rats were anesthetized with an overdose of sodium pentobarbital (90 mg/kg) and perfused intracardially with PBS followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Free-floating sections were processed for TH-immunohistochemistry. Unbiased sterological cell counting procedures were used to count TH-positive cells in the SNpc by using the optical fractionator method in combination with the dissector principle and unbased counting rules (West et al.,1991, *Anat. Rec.* **231,** 482-497; Mouton et al., 2002, *Brain Res.* **956,** 30-35). The entire SNpc was analyzed with a Stereo Investigator Platform (MicroBrightField, Germany) attached to an Olympus BX51 microscope. The loss of TH-positive cells in the SNpc PBS-6-OHDA group and treatment group was 30% and 4%, respectively. Thus, the immunohistochemical analysis showed significant protection of dopaminergic cells by MANF2.

Retrograde Transport

Iodinated MANF2 was injected by means of stereotaxic injection to dorsal pentobarbital (90mg/kg) and perfused intracardially with PBS, followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The brain was removed and it was sliced in 1 mm thick coronal sections. Three millimeter in diameter punctures were taken from dorsal striatum, frontal cortex, hippocampus and substantia nigra. Radioactivity in the punctures was measured by gamma counter (Perkin Elmer). Some of the brains were cut in 40 mM coronal sections and

the slices were placed to autoradiography on X-ray film. Retrograde transport of MANF2 from the dorsal striatum to the substantia nigra was observed in male Wistar rats.

Conclusion.

Our findings indicate that MANF2 can rescue midbrain dopaminergic neurons *in vivo* in the rat 6-OHDA model of Parkinson's disease. MANF2 may, therefore, be used as a therapeutic protein or as a basis for the development of drugs from treatment of Parkinson's disease.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: Helsinki, June 29, 2006

By Mart Saarma, professor
(name of Declarant)

INSTITUTE OF BIOTECHNOLOGY
UNIVERSITAS HELSINGIENSIS

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Saarma, Mart	Director, Professor
eRA COMMONS USER NAME	Institute of Biotechnology, University of Helsinki, Finland

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Tartu University, Estonia	M.Sc.	1972	biochemistry
Tartu University, Estonia	PhD	1975	molecular biology
Institute of Molecular Biology, Russian Academy of Sciences, Moscow	Dr.of Sci.	1986	molecular biology

NOTE: The Biographical Sketch may not exceed four pages. Items A and B (together) may not exceed two of the four-page limit. Follow the formats and instructions on the attached sample.

A. Positions and Honors.

Positions and Employment

- *Research assistant & junior researcher,* Dept. Biochemistry, Tartu State University 1971-1977
- *Head of the Laboratory of Molecular Genetics*
- Institute of Physics, Estonian Academy of Sciences (Tartu) 1977-1980
- *Head of the Department of Molecular Genetics,* Institute of Chemical
- Physics and Biophysics, Estonian Academy of Sciences (Tallinn) 1980-1990
- *Professor,* Gene Technology Center, Tallinn Technical University (part time) 1997-2002
- *Director, Professor,* Institute of Biotechnology, University of Helsinki 1990-

Other Experience and Professional Memberships

- Biocentrum Helsinki-*Vice-chairman of the Board* 1994-
- Scientific Advisory Board of Latvian Biomedical Research Centre
- *-Member of the Board* 1993-
- Committee of equipment of the University of Helsinki-*Chairman* 1997-2005

Institute of Medical Technology, University of Tampere,
-Member of the scientific advisory board 1998-

- Board of the Directors of the biotech company Mobidiag Ltd., Finland

- Member of the Board 2000-

- Board of the Directors of the Finnish Genome Centre

-Chairman of the board 2004-

- Estonian PMs Council for Science and Technology

-Member of the Council 2001-

- Scientific Advisory Board of the Heidelberg Neuroscience Center

-Chairman *of the board* 2002-

- Scientific Board of the Finnish National Public Health Institute

-Member of the board	2002-
• Scientific Advisory Board of the Göttingen Neuroscience Center	
-Member of the board	2003-
• Scientific Advisory Board of the Helsinki Institute of Information Technology	
-Member of the board	2003-
• Board of the Directors of the MobiDiag Ltd.	
-Member of the board	2004
• Board of the Directors of the National Institute of Chemical Physics and Biophysics, Estonia	
-Chairman of the Board	2004-
Morris K. Udall Parkinson's Disease Center of Excellence	
- Member of the External Advisory Board	2005
• Journal Experimental Neurology-*member of the editorial board*	2003-
• Scientific Advisory Board of the Neurotrophics Inc., Canada	
-Member of the scientific advisory board	2000-

Honors

• *Academician,* Estonian Academy of Sciences	1990
• First order decoration of the Finnish White Rose Knighthood	1999
• Foreign Member of the Finnish Academy of Science	2000
• Member of the Tanner Academy	2001
• Second order decoration of the Estonian White Star	2001
• Helsinki Gold medal	2002
• Member of the Finnish Technical Academy of Sciences	2003
• Honorary Member of the Finnish Agricultural Science Foundation	2004

Awards

• Russian Academy of Sciences, prize for the young scientist	1974
• Estonian State prize for science and technology	1980
• Fellow of the Biocentrum Helsinki	1994, 2001
• Member of the Academy of Finland Centre of Excellence in molecular neurobiology	1999-2005
• Finnish Cultural Foundation Science Prize	2000
• Finnish Innovation Prize	2000
• Väino Tanner Prize	2001
• Helsinki Gold medal	2002
• Runeberg Medical Science Prize	2003
• Karl Schlossmann Science Prize	2004

B. Selected peer-reviewed publications (in chronological order).

1. Sariola, H., Saarma, M., Sainio, K., Arumäe, U., Palgi, J., Vaahtokari, A., Thesleff, I. & Karavanov, A. (1991) Dependence of kidney morphogenesis on the expression of nerve growth factor receptor. **Science**, *254*, 571-573.

2.Pirvola, U., Palgi, J., Ylikoski, J., Lehtonen, E. Arumäe, U. & Saarma. M. (1992) Brain-derived neurotrophic factor and neurotrophin 3 in the peripheral target fields of developing inner ear ganglia. **Proc. Natl. Acad. Sci.**, USA, *89,* 9915-9919.
3. Arumäe, U., Pirvola, U., Palgi, J., Kiema, T.-R., Palm, K., Moshnyakov, M., Ylikoski, J. & Saarma, M. (1993) Neurotrophins and their receptors in rat peripheral trigeminal system during maxillary nerve growth. **J. Cell. Biol.**, *122*, 1053-1065.
4.Timmusk, T., Palm, K., Metsis, M., Reintamm, T., Paalme, V., Saarma, M. & Persson, H. (1993) Multiple promoters direct tissue specific expression of rat BDNF gene. **Neuron**, *10*, 475-489.

5. Pichel, J.G., Shen, L., Sheng, H.Z., Granholm, A.-C., Drago, J., Grinberg, A., Lee, E.J., Huang, S.P., Saarma, M., Hoffer, B.J., Sariola, H. & Wesphal, H. (1996) Defects in enteric innervation and kidney development in mice lacking GDNF. **Nature**, *382*, 73-76.
6. Suvanto, P., Hiltunen, J.O., Arumäe, U., Moshnyakov, M., Sariola, H., Sainio, K. & Saarma, M. (1996) Localization of glial cell line-derived neurotrophic factor (GDNF) mRNA in embryonic rat by in situ hybridization. **Eur. J. Neurosci.**, *8,* 816-822.
7. Trupp, M., Arenas, E., Fainzilber, M., Nilsson, A.-S., Sieber, B.-A., Grigoriou, M., Kilkenny, C., Salaxar-Grueso, E., Pachnis, V., Arumäe, U., Sariola, H., Saarma, M. & Ibañéz, C.F. (1996) Functional receptor for GDNF encoded by the c-*ret* proto-oncogene. **Nature**, *381*, 785-789.
8. Suvanto, P., Wartiovaara, K., Lindahl, M., Arumäe, U., Moshnyakov, M., Horelli-Kuitunen, N., Airaksinen, M. S., Palotie, A., Sariola, H. & Saarma, M. (1997) Cloning, mRNA distribution and chromosomal localization of the gene for glial cell line-derived neurotrophic factor receptor beta, a homologue to GDNFR- alpha. **Hum. Mol. Gen.**, Vol. 6, *8*,1267-1273.
9. Reeben, M., Laurikainen, A., Hiltunen, J.O., Castrén, E. & Saarma, M. (1998) The messenger RNAs for both glial cell line-derived neurotrophic factor receptors, C-*ret* and GDNFRα, are induced in the rat brain in response to kainate-induced excitation. **Neuroscience**, *83*(1):151-159.
10. Kokaia, Z., Airaksinen, M. S., Nanobashvili, A., Larsson, E., Kujamäki, E., Lindvall, O. & Saarma, M. (1999) GDNF family ligands and receptors are differentially regulated after brain insults in the rat. **Eur. J. Neurosci.**, *11*, 1202-1216.
11. Poteriaev, D., Titievsky, A., D., Sun, Y. F., Thomas-Crusells, J., Lindahl, M., Billaud, M., Arumäe, U. & Saarma, M. (1999) GDNF triggers a novel Ret-independent Src–kinase family-coupled signaling via a GPI-linked GDNF receptor α1. **FEBS Letters,** 463, 63-66.

12. Rivera, C., Voipio, J., Payne, J.A., Ruusuvuori, E., Lahtinen, H., Lamsa, K., Pirvola, U., Saarma, M. & Kaila, K. (1999) A K^+/Cl^- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. **Nature 397,** 251-255.

13. Rossi, J., Luukko, K., Poteriaev, D., Laurikainen, A., Sun, Y.F., Laakso, T., Eerikäinen, S., Tuominen, R., Lakso, M., Rauvala, H., Arumäe, U., Pasternack, M., Saarma, M. & Airaksinen, M.S. (1999) Retarded growth and deficits in the enteric and parasympathetic nervous system in mice lacking GFRa2, a functional neurturin receptor. **Neuron 22**, 243-252.
14. Meng, X., Lindahl, M., Hyvönen, M. E., Parvinen, M., de Rooij, D. G., Hess, M. W., Raatikainen-Ahokas, A., Sainio, K., Rauvala, H., Lakso, M., Pichel, J. G. , Westphal, H., Saarma, M. & Sariola, H. (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. **Science**, 287, 1489-1493.

15. Lindahl; M., Poteryaev, D.,Liying, Y., Arumäe, U.,Timmusk, T., Bongarzone, I., Aiello, A., Pierotti, M. A., Airaksinen, M.S. & Saarma, M. (2001). Human GFRα4 is the receptor for persephin, and is selectively expressed in normal and malignant thyroid medullary cells. J. Biol. Chem., 276 (12), 9344-9351.

16. Airaksinen, M. S. & Saarma, M. (2002) GDNF family neurotrophic factors: receptor mechanisms, biological functions and therapeutic utility. **Nature Rev. Neurosci.**, 3, 383-394.

17. Rivera, C., Hong Li, Thomas-Crusells, J., Lahtinen, H., Viitanen, T., Nanobashvili, A., Kokaia, Z., Airaksinen, M. S., Voipio, J., Kaila, K. & Saarma, M. (2002). BDNF-induced TrkB activation down-regulates the K+–Cl- cotransporter KCC2 and impairs neuronal Cl- extrusion. **J. Cell Biol.,** 159: 747-752.

18. Popsueva, A., Poteryaev, D., Arighi, E., Meng, X., Angers-Loustau, A., Kaplan, D., Saarma, M. & Sariola, H. (2003). GDNF promotes tubulogenesis of GFRα1-expressing MDCK cells by Src-mediated phosphorylation of MET receptor tyrosine kinase. **J. Cell Biol.** , 161(1):119-129.

19. Yu, L.- Y., Jokitalo, E., Sun, F.- S., Mehlen, P., Lindholm, D., Saarma, M. and Arumäe U. (2003) GDNF-deprived sympathetic neurons die via a novel nonmitochondrial pathway. **J. Cell Biol.**, 163: 987-997.

20. Leppänen, V.-M., Bespalov, M. M., Runeberg-Roos, P., Puurand, Ü., Merits, A., Saarma, M. and Goldman, G. (2004) The structure of GFRα1 domain 3 reveals a novel fold and new insights into GDNF binding and RET activation. **EMBO J.,** 23(7):1452-1462.

C. Research Support

Ongoing Research Support

European Union FP5 research grant QLG-CT-2002-01000 for the topic: "Trophic signaling by GDNF ligands and their receptors in neuronal development and repair" for 2002-2005. M. Saarma: member of the consortium and PI of the Helsinki team.

Academy of Finland Center of Excellence. Special funding for the topic: "Molecular Neurobiology" for 1999-2005. M. Saarma: member of the consortium.

Academy of Finland Research Program in Systems Biology. Funding for 2004-2007 for the project Nro. 1105237 in topic: "Systems level architecture of GDNF mediated neurotrophic action". M. Saarma: PI.

Biocentrum Helsinki, Ministry of Education special biotechnology funding for years 2001-2006 on the topic: " Neurotrophic factors, their receptor and signaling pathways. M. Saarma: PI.

Sigrid Juselius Medical Research Foundation special funding for Mart Saarma and Hannu Sariola for 2003-2008 on the topic: "New GDNF receptors, novel neurotrophic factors and their signalling pathways"- M. Saarma: Co-PI.

Academy of Finland Postdoctoral program. Fellowship Nro. 1209071 for hiring a postdoc in 2004-2007 on a topic: "Novel neurotrophic factors and their receptors". M. Saarma: PI.

Academy of Finland Postdoctoral program. Fellowship for hiring a postdoc in 2005-2008 on a topic: "Studies on the *in vivo* function of GDNF and its receptors". M. Saarma: PI.

National Technology Agency of Finland. Research and Development Project for 2005-2006 Nro 40326/05 : "Search for novel neuroprotective substances". M. Saarma: Co-PI.

Completed Research Support

European Union FP5 research grant QLG3-CT-2000-01405 for the topic: "Cystatin B in epilepsy 2000-2004. M. Saarma: member of the consortium.

Life 2000 Research Program/The Academy of Finland. Funding for the 2000-2003 for the project Nro. 172634 : "Neurotrophic factors and GABA: cross talk in brain development and plasticity". M. Saarma: PI.

Life 2000 Research Program/The Academy of Finland. Funding for the 2000-2003 for the project Nro. 172651: "Integration of cellular lipid dynamics and signaling in neuronal cells". M. Saarma: Co-PI

National Technology Agency Drug 2000 Program. TEKES Project 40296/03 for years 2001-2004:"Novel diagnostic and therapeutic methods based on RET-signaling.

Research collaboration agreement with Contral Pharma Inc., Finland for years 2001-2003 for the topic: "Neurotrophic factors and addiction". M. Saarma: PI.

Research collaboration agreement with Amgen Inc., USA for years 2003-2004 on the topic: "Death pathways activated in cultured neurons by GDNF deprivation." M. Saarma: PI.